3M Specialty Materials

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AR 226 – 1199

90PP

January 3, 2003

FYI-0103-01378

VIA FEDERAL EXPRESS

Document Control Office (DCO)
Office of Pollution Prevention and Toxics (OPPT)
US Environmental Protection Agency
EPA East, Room 6428
1201 Constitution Avenue, NW
Washington, DC 20460

ML#66017

Attention: Docket No. AR-226 and the FYI Docket

Dear Sir or Madam:

This continues 3M's voluntary submissions of data on perfluorooctane sulfonates and related compounds, as part of our ongoing dialogue with EPA regarding fluorochemicals.

Included in this submission are the following:

"Perfluorooctanesulfonyl Fluoride (POSF), T-7661.2, Bacterial Reverse Mutation Test", Huntingdon Life Sciences Ltd., October 23, 2002.

"Perfluorooctanesulfonyl Fluoride (POSF), T-7661.3, *In Vitro* Mammalian Chromosome Aberration Test in Human Lymphocytes, Huntingdon Life Sciences Ltd., October 22, 2002 and Protocol.

We will continue to provide information as it becomes available. Please feel free to contact me if you have any questions.

Very truly yours,

Michael A. Santoro

Michael A. Santoro
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CONTAIN NO CBI

MIN 314/022208

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

BACTERIAL REVERSE MUTATION TEST

Sponsor

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3M Corporate Toxicology
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Research Laboratory

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Report issued 23 October 2002

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice Standards, with the exceptions stated below, and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No. 3106).

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No. L 77/8).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

In line with normal practice in this type of short-term study, the protocol did not require analysis of the dose form.

The expiry date of the test sample was the Sponsor's responsibility.

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Kenneth May, B.Sc., C.Biol., M.I.Biol.,
Study Director,
Huntingdon Life Sciences Ltd.

23 October 2002

Date

QUALITY ASSURANCE STATEMENT

The following inspections and audits have been carried out in relation to this study:

Study Phase	Date of Inspection	Date of Reporting
Protocol Audit	16 October 2001	16 October 2001
Process Based Inspections		
S9 Preparation	15 January 2002	15 January 2002
Formulation and Treatment	31 October 2001	31 October 2001
Plate Scoring	24 October 2001	24 October 2001
Report Audit	4 February 2002	5 February 2002

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Process Based Inspections: At or about the time this study was in progress inspections of routine and repetitive procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated above.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results of this study to reflect the raw data.

A M Jennings — 23 October 2002

Angela M. Jennings, B.Sc., M.Sc., Ph.D., M.R.Q.A., — Date
Group Manager,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

RESPONSIBLE PERSONNEL

Kenneth May, B.Sc., C.Biol., M.I.Biol.
Study Director

Elizabeth Farrall, B.Sc.
Scientist

SUMMARY

In this *in vitro* assessment of the mutagenic potential of Perfluorooctanesulfonyl fluoride (POSF), histidine dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100, and a tryptophan dependent mutant of *Escherichia coli*, strain WP2*uvr*A/pKM101 (CM891), were exposed to the test substance.

Two independent mutation tests were performed in the presence and absence of liver preparations from Aroclor 1254-induced rats (S9 mix). Both tests involved a pre-incubation stage in airtight vessels.

Concentrations of Perfluorooctanesulfonyl fluoride (POSF) up to 5000 µg/plate were tested in the mutation tests. This is the standard limit concentration recommended in the regulatory guidelines that this assay follows. No signs of toxicity were observed towards the tester strains in either mutation test.

No evidence of mutagenic activity was seen at any concentration of Perfluorooctanesulfonyl fluoride (POSF) in either mutation test.

The concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations.

It is concluded that, under the test conditions employed, Perfluorooctanesulfonyl fluoride (POSF) showed no evidence of mutagenic activity in this bacterial system.

INTRODUCTION

This report describes a study designed to assess the mutagenic potential of Perfluorooctanesulfonyl fluoride (POSF) in a bacterial system. The study was conducted in compliance with the following guidelines:

OECD Guidelines for the Testing of Chemicals. (1997) Genetic Toxicology: Bacterial Reverse Mutation Test, Guideline 471.

EC Commission Directive 2000/32/EC Annex 4D-B.13/14. Mutagenicity - Reverse mutation test in bacteria. No: L 136/57.

US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5100 Bacterial reverse mutation test. EPA 712-C-98-247.

Japan Ministry of Agriculture, Forestry and Fisheries. (1985) Notification of Director General, Agricultural Production Bureau. NohSan No. 4200.

Joint Directives of J EPA, J MHW and J MITI. (31 October 1997) Kanpoan No. 287, Eisei No. 127 and Kikyoku No. 2 (31 October 1997).

JMHW Genotoxicity Testing Guideline, PAB Notification No. 1604 (1 November 1999).

Official Notice of J MOL. (8 February 1999).

The method described was also designed to comply with ICH (1995 & 1997), and followed the recommendations of the United Kingdom Environmental Mutagen Society (Gatehouse *et al* 1990).

The *in vitro* technique described by Ames and his co-workers (Ames, McCann and Yamasaki 1975, Maron and Ames 1983) enables the mutagenic effect of a test substance to be determined by exposing specially selected strains of *Salmonella typhimurium* to the test substance. Normally *S. typhimurium* is capable of synthesising the essential amino acid, histidine, but the mutant strains used in this test are incapable of this function. When these strains are exposed to a mutagen, reverse mutation to the original histidine independent form takes place in a proportion of the population. These are referred to as revertants, and are readily detected by their ability to grow and form colonies on a histidine deficient medium (supplemented with biotin, since these strains are also incapable of biotin synthesis).

A technique based on similar principles has also been described by Green (1984). This system employs mutant strains of *Escherichia coli* that are incapable of synthesising the amino acid, tryptophan, which is required for growth.

The strains used carry additional mutations that render them more sensitive to mutagens. The *S. typhimurium* strains have a defective cell coat, which allows greater permeability of test substances into the cell. All the strains are deficient in normal DNA repair processes. In addition, three of them possess a plasmid (pKM101), which introduces an error-prone repair process, resulting in increased sensitivity to some mutagens.

Many substances do not exert a mutagenic effect until they have been metabolised by enzyme systems not available in the bacterial cell. Therefore, the bacteria and test substance are incubated in both the absence and presence of a supplemented liver fraction (S9 mix) prepared from rats previously treated with a substance (Aroclor 1254) known to induce a high level of enzyme activity.

The protocol was approved by Huntingdon Life Sciences Management on 18 July 2001, by the Sponsor on 31 July 2001 and by the Study Director on 15 October 2001.

The study was conducted at Huntingdon Life Sciences Ltd., Eye Research Centre, Eye, Suffolk, IP23 7PX, England.

Experimental start date: 21 November 2001.
Experimental completion date: 31 January 2002.

TEST SUBSTANCE

Identity:	Perfluorooctanesulfonyl fluoride (POSF)
Appearance:	Clear liquid
Storage conditions:	Room temperature
Lot number:	040227
Expiry date:	Sponsor's responsibility; assumed stable for duration of study
Purity:	>95.5%
Specific gravity:	*ca* 1.8
Date received:	14 June 2001

EXPERIMENTAL PROCEDURE

BACTERIAL STRAINS

The following strains were used:

S. typhimurium TA1535: contains a histidine missense mutation (*his*G46) but is also deficient in a DNA repair system (*uvr*B) and has a defective lipopolysaccharide coat on the cell wall (*rfa* mutation). It is reverted by many agents causing base-pair substitutions, but is not sensitive to frameshift mutagens.

S. typhimurium TA100: is the same as TA1535 but contains a resistance transfer factor conferring ampicillin resistance and increasing sensitivity to some mutagens (plasmid pKM101). In addition to base-pair substitutions, it is also able to detect certain frameshift mutagens.

S. typhimurium TA1537: bears a histidine frameshift mutation (*his*C3076). Like TA1535, it is defective in a DNA repair system and lipopolysaccharide coat. It is sensitive to agents causing frameshift mutations involving insertion or deletion of a single base-pair.

S. typhimurium TA98: contains another histidine frameshift mutation (*his*D3052). Again it has a defective DNA repair system and lipopolysaccharide coat but also contains the pKM101 plasmid. It is reverted by agents causing deletion of two adjacent base-pairs (double frameshift mutations), but not by simple alkylating agents causing base-pair substitutions.

E. coli WP2*uvr*A/pKM101: (CM891) contains an ochre mutation. It is reverted by many agents causing A-T base-pair substitutions at the *trp*E locus or by G-C base-pair substitutions in transfer RNA loci elsewhere in the chromosome. It is also deficient in a DNA repair system (*uvr*A), and is more readily reverted by certain mutagens than its parent strain WP2. It also contains the pKM101 plasmid.

The strains of *S. typhimurium* were obtained from the National Collection of Type Cultures, London, England.

The strain of *E. coli* was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Batches of the strains were obtained from master stocks held in liquid nitrogen. The test batches were aliquots of nutrient broth cultures and were stored at -80°C. Dimethyl sulphoxide (DMSO) was added to the cultures at 8% v/v as a cryopreservative. Each batch of frozen strain was tested, where applicable, for cell membrane permeability (*rfa* mutation), sensitivity to UV light and the pKM101 plasmid, which confers resistance to ampicillin. The responses of the strains to a series of diagnostic mutagens were also assessed.

For use in tests, an aliquot of frozen culture was added to 25 ml of nutrient broth and incubated, with shaking, at 37°C for 10 hours. These cultures were intended to provide approximately 10^9 cells per ml, which were measured by spreading aliquots (0.1 ml) of a 10^{-6} dilution of the overnight cultures on the surface of plates of nutrient agar and counting the resultant colonies.

POSITIVE CONTROLS

In the absence of S9 mix

Identity:	Sodium azide
CAS No.:	26628-22-8
Supplier:	Sigma Chemical
Lot number:	77H0079
Purity:	min. 99.5%
Appearance:	White powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	0.5 µg/plate for strains TA1535 and TA100

Identity:	9-Aminoacridine
CAS No.:	90-45-9
Supplier:	Sigma Chemical
Lot number:	106F-06681
Purity:	> 97%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	30 µg/plate for strain TA1537

Identity:	2-Nitrofluorene
CAS No.:	607-57-8
Supplier:	Aldrich Chemical Company
Lot number:	80501-24227
Purity:	98%
Appearance:	Beige powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	1 µg/plate for strain TA98

Identity:	2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)
CAS No.:	3688-53-7
Supplier:	Wako Pure Chemical Industries Ltd.
Lot number:	PAE 1151
Purity:	98-102%
Appearance:	Red powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	0.05 µg/plate for strain WP2*uvr*A/pKM101 (CM891)

In the presence of S9 mix

Identity:	2-Aminoanthracene
CAS No.:	613-13-8
Supplier:	Aldrich Chemical Company
Lot number:	52234-024
Purity:	96%
Appearance:	Green powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	2 µg/plate for strain TA1535 10 µg/plate for strain WP2*uvr*A/pKM101 (CM891)

Identity:	Benzo[a]pyrene
CAS No.:	50-32-8
Supplier:	Aldrich Chemical Company
Lot number:	07778-105
Purity:	98%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	5 µg/plate for strains TA1537, TA98 and TA100

PREPARATION OF S9 FRACTION

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Source:	Charles River UK Ltd.
Weight:	<300 g

S9 fraction was prepared from a group of *ca* 10 animals according to the method described by Ames, McCann and Yamasaki (1975). Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intra-peritoneal injection in corn oil at a dosage of 500 mg/kg body weight. On the fifth day after injection, following overnight fasting, the rats were killed by cervical dislocation and their livers aseptically removed.

The following steps were carried out at 0-4°C under aseptic conditions. The livers were placed in 0.15 M KCl (3 ml KCl : 1 g liver) before being transferred to a Potter-Elvehjem homogeniser. Following preparation, the homogenate was centrifuged at 9000 *g* for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80°C or below. Each batch of S9 fraction was tested for sterility and efficacy.

Date of preparation: 17 July 2001 (test 1); 15 January 2002 (test 2)

PREPARATION OF S9 MIX

The S9 mix contained: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADPH (4 mM) and NADH (4 mM). All the cofactors were filter-sterilised before use.

FORMULATION OF TEST SUBSTANCE

The solubility of the test substance was assessed in dimethyl sulphoxide (DMSO), ethanol, acetone and hexane. It was insoluble in DMSO, ethanol and acetone, and was known to be insoluble in water. Although soluble in hexane at 50 mg/ml, the volume of hexane required to administer the test substance at this concentration was found to be too toxic towards the test system. It was, therefore, decided to administer the test substance by direct addition without the use of a solvent.

All concentrations cited in this report are expressed in terms of the Perfluorooctanesulfonyl fluoride (POSF) sample as received.

MUTATION TEST PROCEDURE

First test (range-finding)

The test substance was added to cultures of the five tester strains at seven concentrations. The highest concentration of test substance tested was 5000 µg/plate (obtained by addition of 2.8 µl of the test substance). This is the standard limit concentration recommended in the regulatory guidelines this assay follows. The other concentrations were 2500 µg/plate (1.4 µl), 1750 µg/plate (1.0 µl), 1250 µg/plate (0.7 µl), 700 µg/plate (0.4 µl), 350 µg/plate (0.2 µl) and 175 µg/plate (0.1 µl). Untreated controls and the appropriate positive controls were also included.

Following the addition of the above aliquots of the test substance (or 0.1 ml of positive control solution) to airtight glass vessels, 0.5 ml S9 mix or 0.5 ml 0.1 M phosphate buffer (pH 7.4) was added, followed by 0.1 ml of a 10 hour bacterial culture. The mixtures were incubated at 37°C for 30 minutes with shaking before addition of 2 ml of agar containing histidine (0.5 mM) and tryptophan (0.5 mM). The mixtures were thoroughly shaken and overlaid onto previously prepared Petri dishes containing 25 ml minimal agar. Each Petri dish was individually labelled with a unique code corresponding to a sheet, identifying the contents of the dish. Three Petri dishes were used for each concentration. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test substance, S9 mix and sodium phosphate buffer. All plates were incubated at 37°C for *ca* 72 hours. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted using a Domino automated colony counter.

Any toxic effects of the test substance would be detected by a substantial reduction in revertant colony counts or by the absence of a complete background bacterial lawn. In the absence of any toxic effects the top concentration normally used in the second test would be the same as that used in the first. If toxic effects were observed a lower concentration might be chosen, ensuring that signs of bacterial inhibition are present at the top concentration. Ideally a minimum of three non-toxic concentrations should be obtained. If precipitate were observed on the plates at the end of the incubation period, at least four non-precipitating dose levels should be obtained, unless otherwise justified by the Study Director.

Second test

The second test was an exact repeat of the first test, except that only five concentrations were used. 5000 µg/plate was again chosen as the top concentration.

STABILITY AND FORMULATION ANALYSIS

The stability of the test substance and the stability and homogeneity of the test substance in the test system were not determined as part of this study. Analysis of achieved concentration was not performed as part of this study.

ASSESSMENT OF RESULTS

Acceptance

For a test to be considered valid the mean of the solvent/vehicle control revertant colony numbers for each strain should lie within the 99% confidence limits of the current historical control range of the laboratory unless otherwise justified by the Study Director. The historical range will be maintained as a rolling record over a maximum of five years. Also, the positive control compounds must cause at least a doubling of mean revertant colony numbers over the negative control.

Analysis

The mean number of revertant colonies for all treatment groups will be compared with those obtained for the solvent/vehicle control groups.

Evaluation

If exposure to a test substance produces an increase in revertant colony numbers of at least twice the concurrent solvent/vehicle controls, with some evidence of a positive dose-relationship (increased revertant colony counts at concentrations below that at which the maximal increase is obtained), in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it will be considered to show evidence of mutagenic activity in this test system. No statistical analysis will be performed.

If exposure to a test substance does not produce an increase in revertant colony numbers in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it will be considered to show no evidence of mutagenic activity in this test system. No statistical analysis will be performed.

If the results obtained fail to satisfy the criteria for a clear "positive" or "negative" response, even after the additional testing outlined in the mutation test procedure, the test data may be subjected to analysis to determine the statistical significance of any increases in revertant colony numbers. The statistical procedures used will be those described by Mahon *et al* (1989) and will usually be analysis of variance followed by Dunnett's test. Biological significance should always be considered along with statistical significance. It should be noted that it is acceptable to conclude an equivocal response if no clear results can be obtained.

DEVIATIONS FROM PROTOCOL

Although the protocol indicated that a solvent or vehicle would be employed, it was not possible to obtain a solution or suspension of the test substance that would be compatible with the test system. The test substance was, therefore, added directly to the test system. Since this procedure complies with the test guidelines that this study follows, this deviation does not impact on the integrity of the study.

MAINTENANCE OF RECORDS

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor. Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

RESULTS

The results obtained with Perfluorooctanesulfonyl fluoride (POSF) and positive control compounds are presented in Tables 1 to 10. The mean values quoted have been corrected to the nearest whole number.

The absence of colonies on sterility check plates confirmed the absence of microbial contamination.

The total colony counts on nutrient agar plates (see Tables) confirmed the viability and high cell density of the cultures of the individual organisms.

The mean revertant colony counts for the solvent controls were within the 99% confidence limits of the current historical control range of the laboratory. Appropriate positive control chemicals (with S9 mix where required) induced substantial increases in revertant colony numbers with all strains, confirming sensitivity of the cultures and activity of the S9 mix.

FIRST TEST (RANGE-FINDING)

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to Perfluorooctanesulfonyl fluoride (POSF) at any concentration in either the presence or absence of S9 mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to Perfluorooctanesulfonyl fluoride (POSF). A maximum exposure concentration of 5000 µg/plate was, therefore, selected for use in the second test.

SECOND TEST

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to Perfluorooctanesulfonyl fluoride (POSF) at any concentration in either the presence or absence of S9 mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to Perfluorooctanesulfonyl fluoride (POSF).

CONCLUSION

It is concluded that, under the test conditions employed, Perfluorooctanesulfonyl fluoride (POSF) showed no evidence of mutagenic activity in this bacterial system.

REFERENCES

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TABLE 1

Results obtained with *S. typhimurium* TA98: test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	46	44	56	49	6
4	POSF	(2500 µg/plate)	+	45	41	42	43	2
5	POSF	(1750 µg/plate)	+	48	45	50	48	3
6	POSF	(1250 µg/plate)	+	43	51	50	48	4
7	POSF	(700 µg/plate)	+	48	50	48	49	1
8	POSF	(350 µg/plate)	+	61	35	52	49	13
9	POSF	(175 µg/plate)	+	56	55	55	55	1
10	Untreated		+	41	36	51	43	8
11	POSF	(5000 µg/plate)	-	41	37	37	38	2
12	POSF	(2500 µg/plate)	-	37	38	35	37	2
13	POSF	(1750 µg/plate)	-	43	31	41	38	6
14	POSF	(1250 µg/plate)	-	39	46	49	45	5
15	POSF	(700 µg/plate)	-	34	39	43	39	5
16	POSF	(350 µg/plate)	-	37	42	41	40	3
17	POSF	(175 µg/plate)	-	44	36	21	34	12
18	Untreated		-	30	30	38	33	5
19	Benzo[a]pyrene	(5 µg/plate)	+	566	546	542	551	13
20	2-Nitrofluorene	(1 µg/plate)	-	234	205	209	216	16
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	133	128	129	130	3

* Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

TABLE 2

Results obtained with *S. typhimurium* TA98: test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	43	48	39	43	5
4	POSF	(2500 µg/plate)	+	42	36	36	38	3
5	POSF	(1750 µg/plate)	+	42	42	36	40	3
6	POSF	(1250 µg/plate)	+	36	43	30	36	7
7	POSF	(700 µg/plate)	+	51	42	46	46	5
8	Untreated		+	53	44	49	49	5
9	POSF	(5000 µg/plate)	-	21	39	34	31	9
10	POSF	(2500 µg/plate)	-	34	38	23	32	8
11	POSF	(1750 µg/plate)	-	37	35	35	36	1
12	POSF	(1250 µg/plate)	-	29	31	29	30	1
13	POSF	(700 µg/plate)	-	32	38	23	31	8
14	Untreated		-	38	39	31	36	4
15	Benzo[a]pyrene	(5 µg/plate)	+	449	550	567	522	64
16	2-Nitrofluorene	(1 µg/plate)	-	504	467	443	471	31
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	106	96	117	106	11

* Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 3

Results obtained with *S. typhimurium* TA100: test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	125	169	137	144	23
4	POSF	(2500 µg/plate)	+	148	130	152	143	12
5	POSF	(1750 µg/plate)	+	153	164	154	157	6
6	POSF	(1250 µg/plate)	+	159	141	165	155	12
7	POSF	(700 µg/plate)	+	154	131	114	133	20
8	POSF	(350 µg/plate)	+	159	145	131	145	14
9	POSF	(175 µg/plate)	+	143	159	128	143	16
10	Untreated		+	148	140	131	140	9
11	POSF	(5000 µg/plate)	-	119	131	122	124	6
12	POSF	(2500 µg/plate)	-	125	132	88	115	24
13	POSF	(1750 µg/plate)	-	125	121	139	128	9
14	POSF	(1250 µg/plate)	-	88	118	124	110	19
15	POSF	(700 µg/plate)	-	138	97	136	124	23
16	POSF	(350 µg/plate)	-	119	118	125	121	4
17	POSF	(175 µg/plate)	-	122	122	109	118	8
18	Untreated		-	112	110	141	121	17
19	Benzo[a]pyrene	(5 µg/plate)	+	573	507	568	549	37
20	Sodium azide	(0.5 µg/plate)	-	360	453	471	428	60
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	168	160	173	167	7

* Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

TABLE 4

Results obtained with *S. typhimurium* TA100: test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	131	145	119	132	13
4	POSF	(2500 µg/plate)	+	148	132	148	143	9
5	POSF	(1750 µg/plate)	+	122	154	139	138	16
6	POSF	(1250 µg/plate)	+	147	132	166	148	17
7	POSF	(700 µg/plate)	+	165	125	143	144	20
8	Untreated		+	131	145	131	136	8
9	POSF	(5000 µg/plate)	-	111	118	123	117	6
10	POSF	(2500 µg/plate)	-	126	137	121	128	8
11	POSF	(1750 µg/plate)	-	135	128	147	137	10
12	POSF	(1250 µg/plate)	-	119	128	124	124	5
13	POSF	(700 µg/plate)	-	150	124	125	133	15
14	Untreated		-	143	123	122	129	12
15	Benzo[a]pyrene	(5 µg/plate)	+	752	706	705	721	27
16	Sodium azide	(0.5 µg/plate)	-	528	501	549	526	24
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	109	110	111	110	1

* Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 5

Results obtained with *S. typhimurium* TA1535: test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	14	21	16	17	4
4	POSF	(2500 µg/plate)	+	20	26	16	21	5
5	POSF	(1750 µg/plate)	+	29	22	12	21	9
6	POSF	(1250 µg/plate)	+	14	26	20	20	6
7	POSF	(700 µg/plate)	+	22	23	19	21	2
8	POSF	(350 µg/plate)	+	17	23	20	20	3
9	POSF	(175 µg/plate)	+	14	20	19	18	3
10	Untreated		+	31	28	19	26	6
11	POSF	(5000 µg/plate)	-	21	15	22	19	4
12	POSF	(2500 µg/plate)	-	15	19	14	16	3
13	POSF	(1750 µg/plate)	-	20	14	24	19	5
14	POSF	(1250 µg/plate)	-	17	15	21	18	3
15	POSF	(700 µg/plate)	-	19	16	24	20	4
16	POSF	(350 µg/plate)	-	16	15	19	17	2
17	POSF	(175 µg/plate)	-	21	20	20	20	1
18	Untreated		-	22	20	21	21	1
19	2-Aminoanthracene	(2 µg/plate)	+	282	324	247	284	39
20	Sodium azide	(0.5 µg/plate)	-	231	252	251	245	12
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	184	165	175	175	10

* Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

TABLE 6

Results obtained with *S. typhimurium* TA1535: test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	20	13	13	15	4
4	POSF	(2500 µg/plate)	+	19	15	13	16	3
5	POSF	(1750 µg/plate)	+	12	13	13	13	1
6	POSF	(1250 µg/plate)	+	20	22	14	19	4
7	POSF	(700 µg/plate)	+	21	14	19	18	4
8	Untreated		+	22	19	19	20	2
9	POSF	(5000 µg/plate)	-	17	14	16	16	2
10	POSF	(2500 µg/plate)	-	15	27	16	19	7
11	POSF	(1750 µg/plate)	-	14	16	22	17	4
12	POSF	(1250 µg/plate)	-	21	14	16	17	4
13	POSF	(700 µg/plate)	-	22	16	27	22	6
14	Untreated		-	27	21	19	22	4
15	2-Aminoanthracene	(2 µg/plate)	+	145	137	121	134	12
16	Sodium azide	(0.5 µg/plate)	-	253	237	288	259	26
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	129	129	161	140	18

* Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 7

Results obtained with *S. typhimurium* TA1537: test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	23	22	15	20	4
4	POSF	(2500 µg/plate)	+	26	22	16	21	5
5	POSF	(1750 µg/plate)	+	23	27	21	24	3
6	POSF	(1250 µg/plate)	+	21	27	19	22	4
7	POSF	(700 µg/plate)	+	21	22	26	23	3
8	POSF	(350 µg/plate)	+	26	21	24	24	3
9	POSF	(175 µg/plate)	+	17	24	24	22	4
10	Untreated		+	22	22	26	23	2
11	POSF	(5000 µg/plate)	-	19	15	10	15	5
12	POSF	(2500 µg/plate)	-	15	10	13	13	3
13	POSF	(1750 µg/plate)	-	17	17	10	15	4
14	POSF	(1250 µg/plate)	-	17	19	10	15	5
15	POSF	(700 µg/plate)	-	16	16	12	15	2
16	POSF	(350 µg/plate)	-	13	9	15	12	3
17	POSF	(175 µg/plate)	-	13	13	10	12	2
18	Untreated		-	16	15	13	15	2
19	Benzo[a]pyrene	(5 µg/plate)	+	355	394	360	370	21
20	9-Aminoacridine	(30 µg/plate)	-	489	457	453	466	20
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	103	139	107	116	20

* Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

TABLE 8

Results obtained with *S. typhimurium* TA1537: test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	16	22	23	20	4
4	POSF	(2500 µg/plate)	+	22	16	20	19	3
5	POSF	(1750 µg/plate)	+	24	29	20	24	5
6	POSF	(1250 µg/plate)	+	22	28	15	22	7
7	POSF	(700 µg/plate)	+	23	26	14	21	6
8	Untreated		+	20	19	26	22	4
9	POSF	(5000 µg/plate)	-	9	8	13	10	3
10	POSF	(2500 µg/plate)	-	10	14	8	11	3
11	POSF	(1750 µg/plate)	-	8	9	14	10	3
12	POSF	(1250 µg/plate)	-	10	17	12	13	4
13	POSF	(700 µg/plate)	-	13	9	13	12	2
14	Untreated		-	16	10	15	14	3
15	Benzo[a]pyrene	(5 µg/plate)	+	315	281	238	278	39
16	9-Aminoacridine	(30 µg/plate)	-	269	276	317	287	26
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	124	129	124	126	3

* Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

000026

TABLE 9

Results obtained with *E. coli* WP2*uvr* A/pKM101 (CM891): test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	107	131	87	108	22
4	POSF	(2500 µg/plate)	+	119	136	96	117	20
5	POSF	(1750 µg/plate)	+	126	122	116	121	5
6	POSF	(1250 µg/plate)	+	123	96	119	113	15
7	POSF	(700 µg/plate)	+	122	144	123	130	12
8	POSF	(350 µg/plate)	+	122	138	119	126	10
9	POSF	(175 µg/plate)	+	117	121	119	119	2
10	Untreated		+	123	121	133	126	6
11	POSF	(5000 µg/plate)	-	101	112	99	104	7
12	POSF	(2500 µg/plate)	-	111	82	89	94	15
13	POSF	(1750 µg/plate)	-	132	118	116	122	9
14	POSF	(1250 µg/plate)	-	85	138	85	103	31
15	POSF	(700 µg/plate)	-	110	86	110	102	14
16	POSF	(350 µg/plate)	-	95	74	84	84	11
17	POSF	(175 µg/plate)	-	81	111	73	88	20
18	Untreated		-	88	124	123	112	21
19	2-Aminoanthracene	(10 µg/plate)	+	320	423	405	383	55
20	AF-2†	(0.05 µg/plate)	-	559	529	531	540	17
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	162	188	217	189	28

* Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

† 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide

TABLE 10

Results obtained with *E. coli* WP2*uvr* A/pKM101 (CM891): test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	125	136	122	128	7
4	POSF	(2500 µg/plate)	+	154	122	165	147	22
5	POSF	(1750 µg/plate)	+	126	159	145	143	17
6	POSF	(1250 µg/plate)	+	132	145	172	150	20
7	POSF	(700 µg/plate)	+	184	143	166	164	21
8	Untreated		+	137	144	165	149	15
9	POSF	(5000 µg/plate)	-	147	148	128	141	11
10	POSF	(2500 µg/plate)	-	121	139	155	138	17
11	POSF	(1750 µg/plate)	-	144	131	145	140	8
12	POSF	(1250 µg/plate)	-	131	131	139	134	5
13	POSF	(700 µg/plate)	-	115	110	135	120	13
14	Untreated		-	153	152	140	148	7
15	2-Aminoanthracene	(10 µg/plate)	+	501	465	583	516	60
16	AF-2†	(0.05 µg/plate)	-	1041	899	957	966	71
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	190	175	189	185	8

* Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

† 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide

APPENDIX 1

Historical control data

Presented below are the historical control data from the period 1 April 1997 to 30 September 2001.

Untreated controls

Strain	TA100		TA1535		WP2*uvr*A/pKM101 (CM891)		TA98		TA1537	
S9 mix	-	+	-	+	-	+	-	+	-	+
Minimum	78	81	12	10	79	73	23	29	7	7
Maximum	149	165	33	29	167	200	48	54	23	34
Mean	109	114	18	19	121	134	38	41	12	13
No. of values	309	313	307	311	214	218	311	315	308	312
Standard deviation	16	22	3	3	17	23	4	5	3	5
Upper 99% limit	151	168	33	29	170	204	49	55	23	35
Lower 99% limit	76	78	12	10	76	69	22	28	7	6

Positive controls

Strain	TA100			TA1535			WP2*uvr*A/pKM101 (CM891)			TA98		TA1537	
S9 mix	-	-	+	-	-	+	-	-	+	-	+	-	+
	(a)	(d)	(h)	(b)	(d)	(i)	(c)	(e)	(j)	(f)	(h)	(g)	(h)
Minimum	212	237	263	53	112	63	294	244	188	123	123	39	67
Maximum	860	1327	1350	781	1130	1145	2312	1533	1704	993	1031	1933	543
Mean	405	564	561	205	419	229	1331	679	659	320	517	350	246
No. of values	346	441	808	341	434	796	112	415	549	788	810	569	810
Standard deviation	120	175	189	139	196	114	488	183	250	115	165	337	80

(a) ENNG 3 µg
(b) ENNG 5 µg
(c) ENNG 2 µg
(d) Sodium azide 0.5µg
(e) AF-2 0.05 µg
(f) 2-Nitrofluorene 1 µg
(g) 9-Aminoacridine 30 µg
(h) Benzo[a]pyrene 5 µg
(i) 2-Aminoanthracene 2 µg
(j) 2-Aminoanthracene 10 µg

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APPENDIX 2

Eye Research Centre GLP Compliance Statement 2001

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

29th January 2001

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger J. Alexander
3/4/01

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

000030

Huntingdon Life Sciences

T-7661.2

PROTOCOL

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

BACTERIAL REVERSE MUTATION TEST

Sponsor

3M Center
3M Corporate Toxicology
Building 220-2E-02
St Paul
MN 55133-3220
USA

Research Laboratory

Huntingdon Life Sciences Ltd.
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

000031

Enquiry number: 23923D

Huntingdon Life Sciences

PROTOCOL APPROVAL

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

BACTERIAL REVERSE MUTATION TEST

C. Atterwill
Management
Huntingdon Life Sciences Ltd.

Date 8/07/01

3M Center
Sponsor

Date 31/7/01

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Study Director approval of the protocol is given on the study details page of the protocol once such details have been established and agreed. The completed page will be issued prior to the start of the study.

Enquiry number: 23923D

Huntingdon Life Sciences

T-7661.2

PROTOCOL APPROVAL

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

BACTERIAL REVERSE MUTATION TEST

C. Atterwill
Management
Huntingdon Life Sciences Ltd.

18/07/01
Date

John R. Butenhoff

3M Center
Sponsor

31/7/01
Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Study Director approval of the protocol is given on the study details page of the protocol once such details have been established and agreed. The completed page will be issued prior to the start of the study.

Enquiry number: 23923D

Huntingdon Life Sciences

STUDY DETAILS PAGE

Study number:	MIN/314
Study title:	Bacterial reverse mutation test
Test substance	
Identity:	Perfluorooctanesulfonyl Fluoride (POSF)
Batch number:	040227
Expiry:	Sponsor's responsibility; assumed stable for duration of study
Appearance:	Clear liquid
Storage conditions:	Room temperature
Purity/Assay:	>95.5%
Specific Gravity:	*ca* 1.8
Solvent:	To be determined
Stability of test substance formulation:	Not assessed in this study
Analysis of achieved concentration:	Not assessed in this study
Sponsor's Monitoring Scientist:	John Butenhoff
Head, Department of Genetic Toxicology:	Dr M. Gillian Clare
Study Director:	Mr Kenneth May
Person acting in the temporary absence of the Study Director:	Miss Elizabeth Farrall
Location of study:	Dept. of Genetic Toxicology, Huntingdon Life Sciences Ltd., Eye, Suffolk, IP23 7PX, England
Proposed study dates	
Experimental start:	20 November 2001
Experimental completion:	5 December 2001
Draft report:	4 January 2002

STUDY DIRECTOR APPROVAL OF PROTOCOL

K M May

Kenneth May
Study Director
Huntingdon Life Sciences Ltd.

15 October 2001

Date

CONTENTS

Huntingdon Life Sciences

1. INTRODUCTION

The object of this study is to assess the mutagenic potential of the test substance in a bacterial system. This procedure complies with the following guidelines:

OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: Bacterial Reverse Mutation Test, Guideline 471.

EC Commission Directive 2000/32/EC Annex 4D-B.13/14. Mutagenicity - Reverse mutation test in bacteria. No. L 136/57.

US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5100 Bacterial reverse mutation test. EPA 712-C-98-247.

Japan Ministry of Agriculture, Forestry and Fisheries. (1985) Notification of Director General, Agricultural Production Bureau. NohSan No. 4200.

Joint Directives of JEPA, JMHW and JMITI (31 October 1997).

KANPOAN No. 287
EISEI No. 127
KIKYOKU No. 2 (31 October 1997).

JMHW Genotoxicity Testing Guideline, PAB Notification No. 1604 (1 November 1999).

Official Notice of J MOL (8 February 1999).

The method described is also designed to comply with ICH (1995 & 1997), and follows the recommendations of the United Kingdom Environmental Mutagen Society (Gatehouse *et al* 1990).

The *in vitro* technique described by Ames and his co-workers (Ames, McCann and Yamasaki 1975; Maron and Ames 1983), enables the mutagenic effect of a test substance to be determined by exposing specially selected strains of *Salmonella typhimurium* to the test substance. Normally *S. typhimurium* is capable of synthesising the essential amino acid, histidine, but the mutant strains used in this test are incapable of this function. When these strains are exposed to a mutagen, reverse mutation to the original histidine independent form takes place in a proportion of the population. These are referred to as revertants, and are readily detected by their ability to grow and form colonies on a histidine deficient medium.

A technique based on similar principles has also been described by Green (1984). This system employs mutant strains of *Escherichia coli* which are incapable of synthesising the amino acid tryptophan required for growth.

The strains used carry additional mutations which render them more sensitive to mutagens. The *S. typhimurium* strains have a defective cell coat which allows greater permeability of test substances into the cell. All the strains are deficient in normal DNA repair processes. In addition three of them possess a plasmid (pKM101) which introduces an error-prone repair process, resulting in increased sensitivity to some mutagens.

Many substances do not exert a mutagenic effect until they have been metabolised by enzyme systems not available in the bacterial cell. Therefore the bacteria and test substance are incubated in both the absence and presence of a supplemented liver fraction (S9 mix) prepared from rats previously treated with a substance (Aroclor 1254) known to induce a high level of enzyme activity.

2. EXPERIMENTAL PROCEDURE

Bacterial strains

The following strains will be used:-

S. typhimurium TA1535 *his*G46 *rfa uvr*B

S. typhimurium TA1537 *his*C3076 *rfa uvr*B

S. typhimurium TA98 *his*D3052 *rfa uvr*B pKM101

S. typhimurium TA100 *his*G46 *rfa uvr*B pKM101

E. coli WP2 *trp uvr*A pKM101 (CM891)

The strains of *S. typhimurium* were obtained from the National Collection of Type Cultures, London, England.

The strain of *E. coli* was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Batches of the strains are obtained from master stocks held in liquid nitrogen. The test batches are aliquots of nutrient broth cultures and are stored at -80°C. Dimethyl sulphoxide (DMSO) is added to the cultures at 8% v/v as a cryopreservative. Each batch of frozen strain is tested, where applicable, for cell membrane permeability (*rfa* mutation), sensitivity to UV light and the pKM101 plasmid which confers resistance to ampicillin. The responses of the strains to a series of diagnostic mutagens are also assessed.

For use in tests an aliquot of frozen culture will be added to 25 ml of nutrient broth and incubated, with shaking, at 37°C for 10 hours. These cultures provide approximately 10^9 cells per ml which will be measured by dilution plating.

Positive controls

In the absence of S9 mix

Identity:	Sodium azide
CAS No.:	26628-22-8
Solvent:	DMSO
Concentration:	0.5 µg/plate for strains TA1535 and TA100

Identity:	9-Aminoacridine
CAS No.:	90-45-9
Solvent:	DMSO
Concentration:	30 µg/plate for strain TA1537

Identity:	2-Nitrofluorene
CAS No.:	607-57-8
Solvent:	DMSO
Concentration:	1 µg/plate for strain TA98

Identity:	2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)
CAS No.:	3688-53-7
Solvent:	DMSO
Concentration:	0.05 µg/plate for strain CM891

In the presence of S9 mix

Identity:	2-Aminoanthracene
CAS No.:	613-13-8
Solvent:	DMSO
Concentration:	2 µg/plate for strain TA1535 10 µg/plate for strain CM891

Identity:	Benzo[a]pyrene
CAS No.:	50-32-8
Solvent:	DMSO
Concentration:	5 µg/plate for strains TA1537, TA98 and TA100

Preparation of S9 fraction

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Age:	7-8 weeks
Weight:	<300 g

S9 fraction will be prepared from a group of usually *ca* 10 animals. Mixed function oxidase systems in the rat liver will be stimulated by Aroclor 1254, administered in an appropriate vehicle as a single intraperitoneal injection at a dosage of 500 mg/kg body weight. On the fifth day after injection, following an overnight starvation, the rats will be killed and their livers aseptically removed.

The following steps will be carried out at 0-4°C under aseptic conditions. The livers will be placed in 0.15 M KCl (3 ml KCl : 1 g liver) before being transferred to a homogeniser. Following preparation, the homogenate will be centrifuged at 9000 *g* for 10 minutes. The supernatant fraction (S9 fraction) will be dispensed into aliquots and stored at -80°C or below until required. All batches of S9 fraction will be tested for sterility and efficacy.

Preparation of S9 mix

S9 mix contains: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADPH (4 mM) and NADH (4 mM). All the cofactors will be filter-sterilised before use.

Selection of solvent/vehicle

A suitable solvent will be selected. If solubility cannot be achieved in a suitable solvent, then a vehicle will be chosen in which a suspension of test substance can be prepared. The identity of the solvent or vehicle will be documented.

Mutation test procedure

First test

The test substance will be added to cultures of the five tester strains usually at a minimum of seven concentrations. The highest concentration will usually be 50 mg/ml of the test substance in the chosen solvent/vehicle, which will provide a final concentration of 5000 µg/plate. This is the standard limit dose recommended in the regulatory guidelines this assay follows. Normally the concentrations will be separated by *ca* half-$\log_{10}$ intervals. The negative control will be the chosen solvent/vehicle. The appropriate positive control compounds will also be included.

Aliquots of 0.1 ml of the test solution/suspension will be placed in airtight glass vessels; this volume may be adjusted if necessary for reasons of test substance solubility or solvent/vehicle toxicity. 0.5 ml S9 mix or 0.5 ml 0.1 M phosphate buffer (pH 7.4) will be added, followed by 0.1 ml of a 10 hour bacterial culture. The mixtures will be incubated at 37°C for 30 minutes with shaking before addition of 2 ml of agar containing histidine (0.5 mM) and tryptophan (0.5 mM). The mixtures will be thoroughly shaken and overlaid onto previously prepared Petri dishes containing 25 ml minimal agar. Each Petri dish will be individually labelled with a unique code corresponding to a sheet, identifying the contents of the dish. Three Petri dishes will be prepared for each dose level. If a solvent/vehicle is used for which there are no historical data to demonstrate that it causes no deleterious or mutagenic effects then a set of untreated control plates will also be prepared containing only bacterial culture and S9 mix or phosphate buffer. Plates will also be prepared without the addition of bacteria in order to assess the sterility of the test substance, S9 mix and phosphate buffer. All plates will be incubated at 37°C for 48-72 hours. After this period the appearance of the background bacterial lawn will be examined and revertant colonies counted using an automated colony counter.

Any toxic effects of the test substance will be detected by a substantial reduction in revertant colony counts or by the absence of a complete background bacterial lawn. In the absence of any toxic effects the top concentration used in the second test will be the same as that used in the first. If toxic effects are observed a lower concentration may be chosen. It should be ensured that if a lower concentration is chosen, signs of bacterial inhibition will be present at the top concentration. Ideally a minimum of three non-toxic concentrations should be obtained. If this is not achieved then the first test may be repeated using a more appropriate dose range. If precipitate is observed on the plates at the end of the incubation period, at least four non-precipitating dose levels should be obtained, unless otherwise justified by the Study Director. This should be taken into account when selecting the dose levels for the second assay and may necessitate the repetition of the first test if this requirement has not been met.

Second test

If a clear positive response is obtained in the first test, the second test will be an exact repeat of the first. However, a minimum of only five concentrations of test substance will be used. If a negative or equivocal response is obtained a variation on the above procedure may be used. Variations include (but are not restricted to) different S9 concentrations or narrowing the dose range.

If the required number of non-toxic dose levels is not obtained, the second test may be repeated using a more appropriate dose range. It may also be repeated to confirm a positive response. Additional testing may be performed if no clear response is obtained. The exact design of any additional test will be at the discretion of the Study Director.

3. ASSESSMENT OF RESULTS

Acceptance

For a test to be considered valid the mean of the solvent/vehicle control revertant colony numbers for each strain should lie within the 99% confidence limits of the current historical control range of the laboratory unless otherwise justified by the Study Director. The historical range will be maintained as a rolling record over a maximum of five years. Also, the positive control compounds must cause at least a doubling of mean revertant colony numbers over the negative control.

Analysis

The mean number of revertant colonies for all treatment groups will be compared with those obtained for the solvent/vehicle control groups.

Evaluation

If exposure to a test substance produces an increase in revertant colony numbers of at least twice the concurrent solvent/vehicle controls, with some evidence of a positive dose-relationship (increased revertant colony counts at concentrations below that at which the maximal increase is obtained), in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it will be considered to show evidence of mutagenic activity in this test system. No statistical analysis will be performed.

If exposure to a test substance does not produces an increase in revertant colony numbers in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it will be considered to show no evidence of mutagenic activity in this test system. No statistical analysis will be performed.

If the results obtained fail to satisfy the criteria for a clear "positive" or "negative" response, even after the additional testing outlined in the mutation test procedure, the test data may be subjected to analysis to determine the statistical significance of any increases in revertant colony numbers. The statistical procedures used will be those described by Mahon *et al* (1989) and will usually be analysis of variance followed by Dunnett's test. Biological significance should always be considered along with statistical significance. It should be noted that it is acceptable to conclude an equivocal response if no clear results can be obtained.

4. REPORTING

The report will contain details of the test substance, methodology, results and interpretation of the data. Tabulated results will show individual plate counts, mean revertant colony counts and their standard deviation. Where appropriate, graphs will also be included. Good Laboratory Practice and Quality Assurance statements will be included.

In the absence of ongoing communications, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional cost.

Upon study completion, two types of report are issued:

Draft report: Following QA audit, for review by the Sponsor

Final report: After approval by the Sponsor

Reports will be supplied on A4 paper and the following number of copies will be supplied:

Draft report: 1 unbound (double sided)

Final report: 1 bound (double sided with original signatures)

1 unbound (single sided)

Any additions or corrections to an authorised final report will be documented as a formal amendment to the protocol.

5. MAINTENANCE OF RECORDS

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

6. GOOD LABORATORY PRACTICE

The study will be conducted in compliance with the principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

7. QUALITY ASSURANCE

The following will be inspected or audited in relation to this study.

Protocol Audit	:	Study protocol.
Process based inspections	:	Routine and repetitive procedures will be inspected on representative studies, not necessarily on this study.
Report Audit	:	The draft report and study data will be audited before issue of the draft report to the Sponsor.

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections, which will be reported to appropriate Company Management only.

8. HEALTH & SAFETY

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1994, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

9. REFERENCES

AMES, B.N., McCANN, J. and YAMASAKI, E. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. *Mutation Research*, **31**, 347-364.

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GREEN, M.H.L. (1984) Mutagen testing using *trp*$^+$ reversion in *Escherichia coli* in: KILBEY, B.J., LEGATOR, M., NICHOLS, W. and RAMEL, C. (Eds.). *Handbook of Mutagenicity Test Procedures. Second edition*, pp.161-187. Elsevier Science Publishers BV, Amsterdam.

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MARON, D.M. and AMES, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutation Research*, **113**, 173-215.

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

T-7661.3

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES

Sponsor

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3M Corporate Toxicology
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Report issued 22 October 2002

CONTENTS

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TABLES

APPENDICES

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards, with the exceptions stated below, and I consider the data generated to be valid.

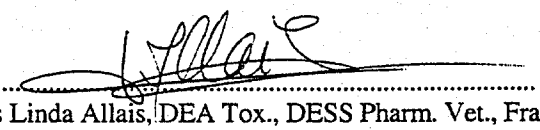
The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No. 3106).

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No. L 77/8).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

In line with normal practice in this type of short-term study, the protocol did not require analysis of the dose form.

The expiry date of the test substance was the responsibility of the Sponsor.



Ms Linda Allais, DEA Tox., DESS Pharm. Vet., France
Study Director,
Department of Genetic Toxicology,
Huntingdon Life Sciences Ltd.

22 October 2002
Date

QUALITY ASSURANCE STATEMENT

The following have been inspected or audited in relation to this study.

Study Phase	Date of Inspection	Date of Reporting
Protocol Audit	11 January 2002	11 January 2002
Study Based Inspection		
Culture treatment	17 January 2002	17 January 2002
Process Based Inspections		
Formulation	14 January 2002	14 January 2002
Culture Establishment	11 February 2002	11 February 2002
Harvesting and slide preparation	7 February 2002	8 February 2002
Slide scoring	5 March 2002	5 March 2002
S-9 preparation	15 January 2002	15 January 2002
Report Audit	15 April 2002	15 April 2002

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Study Based Inspection: An inspection of a phase of this study was conducted and reported to the Study Director and Company Management as indicated above.

Process Based Inspections: At or about the time this study was in progress inspections of routine and repetitive procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated above.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

.....
Angela Jennings, B.Sc., M.Sc., PhD., M.R.Q.A.,
Group Manager,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

22 October 2002
Date

RESPONSIBLE PERSONNEL

Linda Allais, DEA Tox., DESS Pharm. Vet., France,
Study Director,
Department of Genetic Toxicology.

Lincoln Pritchard, B.Sc.,
Study Supervisor,
Department of Genetic Toxicology.

SUMMARY

A study was performed to assess the ability of Perfluorooctanesulfonyl Fluoride (POSF) to induce chromosomal aberrations in human lymphocytes cultured *in vitro*.

Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of S9 mix derived from rat livers. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of Perfluorooctanesulfonyl Fluoride (POSF) to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

First test

With and without S9 mix - 3 hours treatment, 17 hours recovery: 1.25, 2.5 and 5% v/v atmosphere.

Second test

Without S9 mix - 20 hours continuous treatment: 0.8, 1 and 2% v/v atmosphere.

With S9 mix - 3 hours treatment, 17 hours recovery: 2, 5 and 7.5% v/v atmosphere.

In both the absence and presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused no statistically significant increase in the proportion of metaphase figures containing chromosomal aberrations at any dose level, when compared with the solvent control, in either test.

A quantitative analysis for polyploidy was made in cultures treated with the negative control and highest dose level. No statistically significant increases in the proportion of polyploid cells were seen.

All positive control compounds caused large statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It is concluded that the test substance Perfluorooctanesulfonyl Fluoride (POSF) has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described.

INTRODUCTION

This report describes a study designed to assess the ability of Perfluorooctanesulfonyl Fluoride (POSF) to cause chromosomal aberrations in human lymphocytes cultured *in vitro*.

The study was conducted in compliance with the following guidelines:

> OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: *In Vitro* Mammalian Chromosome Aberration Test, Guideline 473.
>
> US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5375 *In Vitro* Mammalian Chromosome Aberration Test. EPA 712-C-98-223.

Human lymphocytes have been used in this type of study for a number of years (Evans and O'Riordan 1975, Scott, Dean, Danford and Kirkland 1990). They are cultured *in vitro* but do not divide unless stimulated to do so. This is achieved by adding phytohaemagglutinin (PHA) to the culture that results in a high mitotic yield (Nowell 1960).

In this study, blood taken from healthy male non-smoking donors was pooled and diluted with tissue culture medium. The cultures were incubated in the presence of PHA before being treated with the test substance. Following treatment the cells were arrested at metaphase using the mitotic inhibitor, Colcemid®. Chromosomes in these metaphase cells were then examined for the presence of chromosome aberrations. The best estimate of the aberration frequency is at the first cell division after initiation of treatment since certain types of damage may be lost during subsequent cell divisions. In this laboratory the cell cycle time for human lymphocytes in whole blood culture is approximately 13-14 hours.

The study was performed on two separate occasions. In the first test, a three hour treatment was used in both the presence and the absence of S9 mix. In the second test, a continuous treatment was used without S9 mix, and the test with S9 mix was a repeat of the first test.

Aberrations were scored according to the classification of the ISCN (1985). Traditionally gaps have been excluded from the quantitation of chromosome aberrations. Some gaps, however, have been shown to be real discontinuities in DNA (Heddle and Bodycote 1970, Satya-Prakash, Hsu and Pathak 1981). In this study the total number of cells containing aberrations both with and without gaps has been calculated.

Many substances do not exert a mutagenic effect until they have been metabolised by enzyme systems that are not found in cultured cells. Therefore the cultures and test substance were incubated in both the absence and presence of a supplemented liver fraction (S9 mix) prepared from rats previously treated with a substance (Aroclor 1254) known to induce a high level of enzymic activity (Maron and Ames 1983, Natarajan *et al.* 1976).

The protocol was approved by Huntingdon Life Sciences Management on 18 July 2001, by the Sponsor on 31 August 2001 and by the Study Director on 10 January 2002.

The study was conducted at Huntingdon Life Sciences Ltd., Eye, Suffolk, IP23 7PX, England.

The experimental start and completion dates of the study were 15 January 2002 and 15 March 2002, respectively.

TEST SUBSTANCE

Identity:	Perfluorooctanesulfonyl Fluoride (POSF)
CAS No.:	307-35-7
Appearance:	Clear liquid
Storage conditions:	Room temperature
Batch number:	040227
Expiry:	Sponsor's responsibility; assumed stable for the duration of the study
Purity:	>95.5 %
Date received:	14 June 2001

EXPERIMENTAL PROCEDURE

CULTURE OF LYMPHOCYTES

Human blood was collected aseptically from healthy, non-smoking male donors, pooled and diluted with RPMI 1640 tissue culture medium supplemented with 10% foetal calf serum, 1 unit/ml Heparin, 20 I.U./ml penicillin/20 μg/ml streptomycin and 2.0 mM glutamine. Aliquots (0.4 ml blood : 4.5 ml medium : 0.1 ml phytohaemagglutinin) of the cell suspension were placed in sterile universal containers and incubated at 37°C for approximately 48 hours. The cultures were gently shaken daily to resuspend the cells.

POSITIVE CONTROLS

In the absence of S9 mix

Identity:	Mitomycin C
Supplier:	Sigma Chemical Co Ltd
Appearance:	Blue powder
Batch number:	31K2500
Solvent:	Sterile purified water
Final concentration:	0.2 μg/ml (3 hour treatment) 0.1 μg/ml (continuous treatment)

In the presence of S9 mix

Identity:	Cyclophosphamide
Supplier:	Asta Medica Ltd
Appearance:	White powder
Batch number:	ON465 (Test 1) and 1H485 (Test 2)
Solvent:	Sterile purified water
Final concentration:	10 μg/ml

PREPARATION OF S9 FRACTION

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Source:	Charles River UK
Age:	7 - 8 weeks
Weight:	<300 g

S9 fraction was prepared from a group of *ca.* 10 animals. Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in corn oil at a dosage of 500 mg/kg bodyweight. On the fifth day after injection, following an overnight starvation, the rats were killed and their livers aseptically removed.

The following steps were carried out at 0 - 4°C under aseptic conditions. The livers were placed in 0.15 M KCl (3 ml KCl : 1 g liver) before being transferred to an homogeniser. Following preparation, the homogenates were centrifuged at 9000 *g* for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80°C or below until required.

PREPARATION OF S9 MIX

S9 mix contained: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium orthophosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter-sterilised before use.

TREATMENT OF CELLS WITH TEST SUBSTANCE - FIRST TEST

After approximately 48 hours, the cultures were centrifuged and the cells were resuspended in fresh culture medium. Atmospheres of Perfluorooctanesulfonyl Fluoride (POSF) were established in sealed glass bottles (160 ml internal volume) with septum caps to give final concentrations of 1.25, 2.5, 5, 10, 20, 40 and 70% v/v atmosphere. Air was withdrawn from each bottle and then an appropriate volume of Perfluorooctanesulfonyl Fluoride (POSF) was introduced using a syringe and needle, inserted through the septum cap. After evaporation of Perfluorooctanesulfonyl Fluoride (POSF) and equilibration of the atmospheres at 37 °C, the lymphocyte cultures were injected into the bottles. The glass bottles were then incubated on their sides at 37 °C in a roller apparatus (see appendix 3: Apparatus for vapour/gas phase exposure of cultivated mammalian cells), which rotates the bottles once every eight minutes approximately. The lymphocytes coat the inside of the bottles and were immersed in culture medium once every revolution and exposed directly to Perfluorooctanesulfonyl Fluoride (POSF) for the rest of the revolution. The solvent control (Air) was established in duplicate cultures and contained an atmosphere of air. Mitomycin C, at a final concentration of 0.2 μg/ml, was added to duplicate cultures and also contained an atmosphere of air.

Immediately before treatment of the second set of cultures, 1 ml of medium was removed from each culture and discarded. This was replaced with 1 ml of S9 mix. The cultures were then added to the appropriate glass bottle giving the same series of final concentrations as above. The duplicate solvent control cultures were established under an atmosphere of air. Cyclophosphamide was added to duplicate cultures at a final concentration of 10 μg/ml that were contained in an atmosphere of air.

Three hours after dosing, the cultures were centrifuged at 500 *g* for 5 minutes. The cells were rinsed and resuspended in fresh medium under an atmosphere of air in universal containers. They were then incubated for a further 17 hours.

HARVESTING AND FIXATION

Two hours before the cells were harvested, mitotic activity was arrested by addition of Colcemid® (Sigma) to each culture at a final concentration of 0.1 µg/ml. After 2 hours incubation, each cell suspension was transferred to a centrifuge tube and centrifuged for 5 minutes at 500 *g*. The cell pellets were treated with a hypotonic solution (0.075M KCl prewarmed at 37°C). After a 10 minute period of hypotonic incubation at 37°C, the suspensions were centrifuged at 500 *g* for 5 minutes and the cell pellets fixed by addition of freshly prepared cold fixative (3 parts methanol : 1 part glacial acetic acid). The fixative was replaced further times until it became colourless.

SLIDE PREPARATION

The pellets were resuspended, then centrifuged at 500 *g*. for 5 minutes and finally resuspended in a small volume of fresh fixative. A few drops of the cell suspensions were dropped onto pre-cleaned microscope slides which were then allowed to air-dry. The slides were then stained in 10% Giemsa, prepared in buffered water (pH 6.8). After rinsing in buffered water the slides were left to air-dry and then mounted in DPX. The remaining cultures in fixative were stored at 4°C until slide analysis was completed.

MICROSCOPIC EXAMINATION

The prepared slides were examined by light microscopy using a low power objective. The proportion of mitotic cells per 1000 cells in each culture was recorded except for positive control treated cultures. From these results the dose level causing a decrease in mitotic index of approximately 50% of the solvent control value or, if there was no decrease, the maximum achievable concentration was used as the highest dose level for the metaphase analysis. The intermediate and low dose levels were also selected.

The concentration of each positive control compound selected for analysis was the lowest concentration dosed unless a preliminary scan of metaphase figures indicated an insufficient level of aberrant cells.

The selected slides were then coded. Metaphase cells were identified using a low power objective and examined at a magnification of x1000 using an oil immersion objective. One hundred metaphase figures were examined, where possible, from each culture. This number was reduced in cultures showing a high level of aberrant cells. Chromosome aberrations were scored according to the classification of the ISCN (1985). Only cells with 44 - 48 chromosomes were analysed. Polyploid and endoreduplicated cells were noted when seen. The vernier readings of all aberrant metaphase figures were recorded.

The incidence of polyploid metaphase cells, out of 500 metaphase cells, was determined quantitatively for negative control cultures and cultures treated with the highest dose level of the test substance used in the analysis for chromosomal aberrations.

The number of aberrant metaphase cells in each treatment group was compared with the solvent control value using Fisher's test (Fisher 1973).

SECOND TEST

Cultures were initiated and maintained as previously described. In this second test a continuous treatment was used in the absence of S9 mix. In the presence of S9 mix, a three hour treatment was used, as in the first test. The harvest time was at 20 hours for both parts of the test. Concentrations of Perfluorooctanesulfonyl Fluoride (POSF) were as follows:

Without S9 mix: 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2 and 5% v/v atmosphere.

With S9 mix: 0.6, 0.8, 1, 2, 5 and 7.5% v/v atmosphere.

Duplicate cultures were used for each treatment and the solvent control. Mitomycin C, at a final concentration of 0.1 µg/ml, and Cyclophosphamide, at a final concentration of 10 µg/ml, were added to duplicate cultures.

Three hours after dosing, the cultures containing S9 mix were centrifuged. The cells were rinsed and resuspended in fresh medium under an atmosphere of air in universal containers. They were then incubated for a further 17 hours. Cultures treated in the absence of S9 mix were incubated for 20 hours.

All cultures were treated with Colcemid®, at a final concentration of 0.1 µg/ml, two hours before the end of the incubation period. They were then harvested, fixed and the slides prepared as previously described. The slides were then examined microscopically as previously described.

STABILITY, HOMOGENEITY AND FORMULATION ANALYSIS

The stability and homogeneity of the test substance and of the test substance in the solvent were not determined as part of this study. Analysis of achieved concentration was not performed as part of this study.

ASSESSMENT OF RESULTS

An assay is considered to be acceptable if the negative and positive control values lie within the current historical control range.

The test substance is considered to cause a positive response if the following conditions are met:

Statistically significant increases ($P<0.01$) in the frequency of metaphases with aberrant chromosomes (excluding gaps) are observed at one or more test concentration.

The increases exceed the negative control range of this laboratory, taken at the 99% confidence limit.

The increases are reproducible between replicate cultures.

The increases are not associated with large changes in osmolality of the treatment medium or extreme toxicity.

Evidence of a dose-relationship is considered to support the conclusion.

A negative response is claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies are observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

MAINTENANCE OF RECORDS

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

RESULTS

FIRST TEST

Toxicity data

Mitotic indices of cultured human lymphocytes treated with Perfluorooctanesulfonyl Fluoride (POSF) are shown in Table 2.

In the absence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused a reduction in the mitotic index to 57% of the solvent control value at dose level of 5% v/v atmosphere. The dose levels selected for the metaphase analysis were 1.25, 2.5 and 5% v/v atmosphere.

In the presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused a reduction in the mitotic index to 52% of the solvent control value at dose level of 5% v/v atmosphere. The dose levels selected for the metaphase analysis were 1.25, 2.5 and 5% v/v atmosphere.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase figures when compared to the solvent control.

Metaphase analysis

The effects of Perfluorooctanesulfonyl Fluoride (POSF) on the chromosomes of cultured human lymphocytes are shown in Table 3 and summarised in Table 1.

In both the absence and the presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused no statistically significant increase in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, Mitomycin C and Cyclophosphamide, caused large statistically significant increases ($P<0.001$) in the proportion of aberrant cells. This demonstrated the efficacy of the S9 mix and the sensitivity of the test system.

SECOND TEST

Toxicity data

Mitotic indices of cultured human lymphocytes treated with Perfluorooctanesulfonyl Fluoride (POSF) are shown in Table 4.

In the absence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused a reduction in the mitotic index to 61% of the solvent control value at dose level of 2% v/v atmosphere. The dose levels selected for the metaphase analysis were 0.8, 1 and 2% v/v atmosphere.

In the presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused a reduction in the mitotic index to 39% of the solvent control value at dose level of 7.5% v/v. The dose levels selected for the metaphase analysis were 2, 5 and 7.5% v/v.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase cells when compared to the solvent control.

Metaphase analysis

The effects of Perfluorooctanesulfonyl Fluoride (POSF) on the chromosomes of cultured human lymphocytes are shown in Table 5 and summarised in Table 1.

In both the absence and the presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused no statistically significant increase in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, Mitomycin C and Cyclophosphamide, caused large, statistically significant increases ($P<0.001$) in the proportion of aberrant cells.

CONCLUSION

It is concluded that the test substance Perfluorooctanesulfonyl Fluoride (POSF) has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described.

REFERENCES

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TABLE 1

Summary of Results

Test 1

Exposure period (hours)	S9 mix	Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Cells with aberrations Excluding gaps			Cells with aberrations Including gaps			Relative Mitotic Index (%)
			Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
3	-	0 (Air)	1	1	1.0	2	2	2.0	100
		1.25	5	4	4.5	5	5	5.0	60
		2.5	3	0	1.5	3	2	2.5	72
		5	5	1	3.0	7	1	4.0	57
		0.2 μg/ml (Mitomycin C)	[a] 18	[a] 24	21.0***	[a] 24	[a] 28	26.0***	-
3	+	0 (Air)	2	2	2.0	4	4	4.0	100
		1.25	1	3	2.0	1	5	3.0	92
		2.5	3	5	4.0	3	6	4.5	80
		5	1	1	1.0	1	2	1.5	52
		10μg/ml (Cyclophosphamide)	[a] 20	23	22.0***	[a] 22	29	26.7***	-

Test 2

Exposure period (hours)	S9 mix	Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Cells with aberrations Excluding gaps			Cells with aberrations Including gaps			Relative Mitotic Index (%)
			Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
20	-	0 (Air)	2	0	1.0	2	0	1.0	100
		0.8	0	2	1.0	0	3	1.5	69
		1	2	1	1.5	3	1	2.0	66
		2	0	0	0.0	0	0	0.0	61
		0.1 μg/ml (Mitomycin C)	9	7	8.0***	9	7	8.0***	-
3	+	0 (Air)	1	1	1.0	2	2	2.0	100
		2	1	1	1.0	3	2	2.5	73
		5	0	1	0.5	1	2	1.5	70
		7.5	[b] 0	1	0.7	[b] 0	2	1.3	39
		10μg/ml (Cyclophosphamide)	[a] 18	[a] 18	18.0***	[a] 20	[a] 20	20.0***	-

*** P<0.001
Otherwise P≥0.01

[a] 50 cells were analysed from these cultures due to high levels of aberrations seen
[b] 50 cells were analysed from this culture due to insufficient metaphases present on slide

TABLE 2

Mitotic index data - first test

Without S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Mitotic index #		Relative mitotic index #	Polyploidy	
	Incidence	% Mean	(%)	Incidence	% Mean
0 (Air)	88/1000 92/1000	9.0	100	1/500 1/500	0.2
1.25	51/1000 57/1000	5.4	60		
2.5	74/1000 55/1000	6.5	72		
5	57/1000 45/1000	5.1	57	2/500 0/500	0.2
10	a a				
20	b b				
40	b b				
70	b b				

\# Calculations have been made using rounded values
a Very few metaphases present on slide
b No cells, no metaphases present on slide

TABLE 2

Mitotic index data - first test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Mitotic index #		Relative mitotic index #	Polyploidy	
	Incidence	% Mean	(%)	Incidence	% Mean
0 (Air)	97/1000 100/1000	9.9	100	1/500 1/500	0.2
1.25	68/1000 113/1000	9.1	92		
2.5	64/1000 93/1000	7.9	80		
5	76/1000 26/1000	5.1	52	0/500 0/500	0.0
10	a a				
20	b b				
40	b b				
70	b -				

\# Calculations have been made using rounded values
a Very few live cells, no metaphases present on slide
b No cells, no metaphases present on slide
\- Not assessed

TABLE 3

Metaphase analysis data - first test

Without S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	No. cells examined	Aberrations				No. of aberrant cells				Relative Mitotic index %
		Chromatid type	Chromosome type	Others	Gaps	Exc.	Mean	Inc.	Mean	
		ctb cte	csb cse		ctg csg	gaps	%	gaps	%	
0	100	1			1	1	1.0	2	2.0	100
(Air)	100	1			1	1		2		
1.25	100	5				5	4.5	5	5.0	60
	100	2	2		1	4		5		
2.5	100	5				3	1.5	3	2.5	72
	100				2	0		2		
5	100	4	1		2	5	3.0	7	4.0	57
	100	1				1		1		
0.2 μg/ml	50	8	2		4	9	21.0	12	26.0	-
(Mitomycin C)	50	13	3		1 1	12	***	14	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

***	P<0.001
Otherwise	P≥0.01

TABLE 3

Metaphase analysis data - first test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	No. cells examined	Aberrations					No. of aberrant cells				Relative Mitotic index %
		Chromatid type		Chromosome type		Others	Gaps	Exc. gaps	Mean %	Inc. gaps	Mean %	
		ctb	cte	csb	cse		ctg csg					
0	100	2					3	2	2.0	4	4.0	100
(Air)	100	2					2	2		4		
1.25	100	1						1	2.0	1	3.0	92
	100	3		1			2	3		5		
2.5	100	5					1	3	4.0	3	4.5	80
	100	3		3			2	5		6		
5	100	1						1	1.0	1	1.5	52
	100	1					1	1		2		
10 μg/ml	50	7	1	2			2	10	22.0	11	26.7	-
(Cyclophosphamide)	100	19	2	4			11	23	***	29	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

***	P<0.001
Otherwise	P≥0.01

TABLE 4

Mitotic index data - second test

Without S9 mix, 20 hours continuous treatment

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Mitotic index #		Relative mitotic index #	Polyploidy	
	Incidence	% Mean	(%)	Incidence	% Mean
0 (Air)	126/1000 107/1000	11.7	100	0/500 0/500	0.0
0.1	104/1000 113/1000	10.9	93		
0.2	114/1000 103/1000	10.9	93		
0.4	90/1000 97/1000	9.4	80		
0.6	105/1000 64/1000	8.5	73		
0.8	71/1000 90/1000	8.1	69		
1	72/1000 81/1000	7.7	66		
2	65/1000 77/1000	7.1	61	0/500 0/500	0.0
5	14/1000 29/1000	2.2	19		

Calculations have been made using rounded values

TABLE 4

Mitotic index data - second test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Mitotic index #		Relative mitotic index #	Polyploidy	
	Incidence	% Mean	(%)	Incidence	% Mean
0 (Air)	69/1000 94/1000	8.2	100	0/500 1/500	0.1
0.6	89/1000 74/1000	8.2	100		
0.8	72/1000 79/1000	7.6	93		
1	56/1000 83/1000	7.0	85		
2	60/1000 59/1000	6.0	73		
5	58/1000 56/1000	5.7	70		
7.5	29/1000 35/1000	3.2	39	0/71 0/241	0.0

Calculations have been made using rounded values

TABLE 5

Metaphase analysis data - second test

Without S9 mix, 20 hours continuous treatment

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	No. cells examined	Aberrations				No. of aberrant cells				Relative Mitotic index %
		Chromatid type		Chromosome type		Others	Gaps	Exc. gaps	Mean %	Inc. gaps	Mean %	
		ctb	cte	csb	cse		ctg csg					
0	100	1		2				2	1.0	2	1.0	100
(Air)	100							0		0		
0.8	100							0	1.0	0	1.5	69
	100	2					1	2		3		
1	100	2					1	2	1.5	3	2.0	66
	100			1				1		1		
2	100							0	0.0	0	0.0	61
	100							0		0		
0.1 μg/ml	100	6	3	1				9	8.0	9	8.0	-
(Mitomycin C)	100	3	2	2			1	7	***	7	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

***	P<0.001
Otherwise	P≥0.01

TABLE 5

Metaphase analysis data - second test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

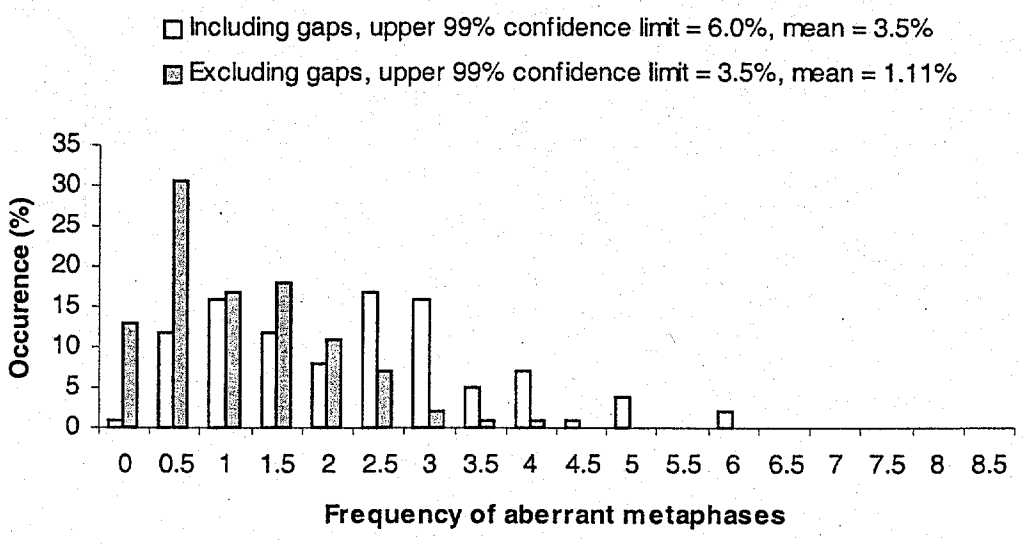
Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	No. cells examined	Aberrations				No. of aberrant cells				Relative Mitotic index %
		Chromatid type		Chromosome type		Others	Gaps	Exc.	Mean	Inc.	Mean	
		ctb	cte	csb	cse		ctg csg	gaps	%	gaps	%	
0	100			1			1	1	1.0	2	2.0	100
(Air)	100	1					1	1		2		
2	100	1					2	1	1.0	3	2.5	73
	100	1					1	1		2		
5	100						1	0	0.5	1	1.5	70
	100	1					1	1		2		
7.5	50							0	0.7	0	1.3	39
	100	1					1	1		2		
10 μg/ml	50	8		3			1	9	18.0	10	20.0	-
(Cyclophosphamide)	50	9	1	2			3 1	9	***	10	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

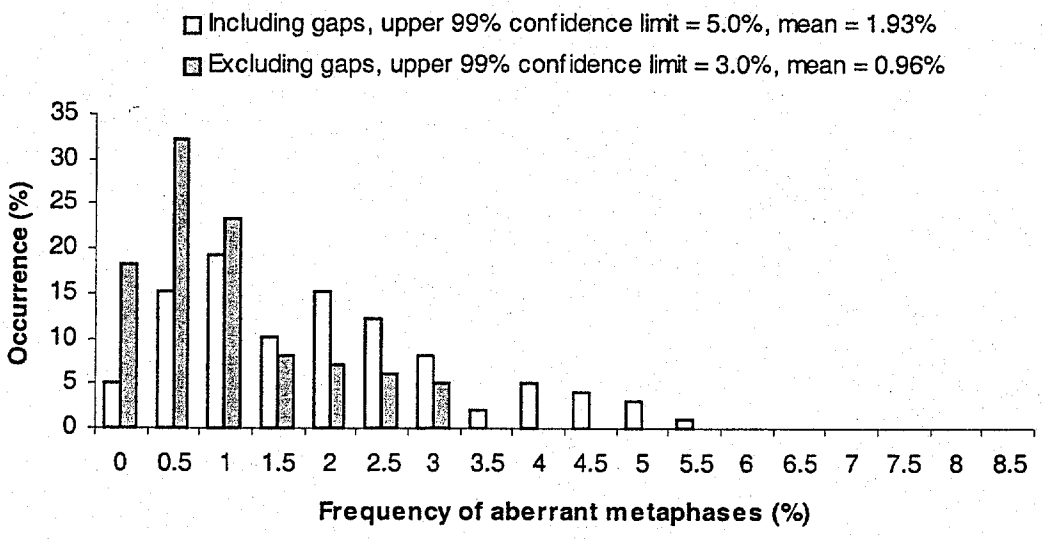
***	P<0.001
Otherwise	P≥0.01

APPENDIX 1

Historical negative control data (January 1999 - December 2001)
Without S9 mix

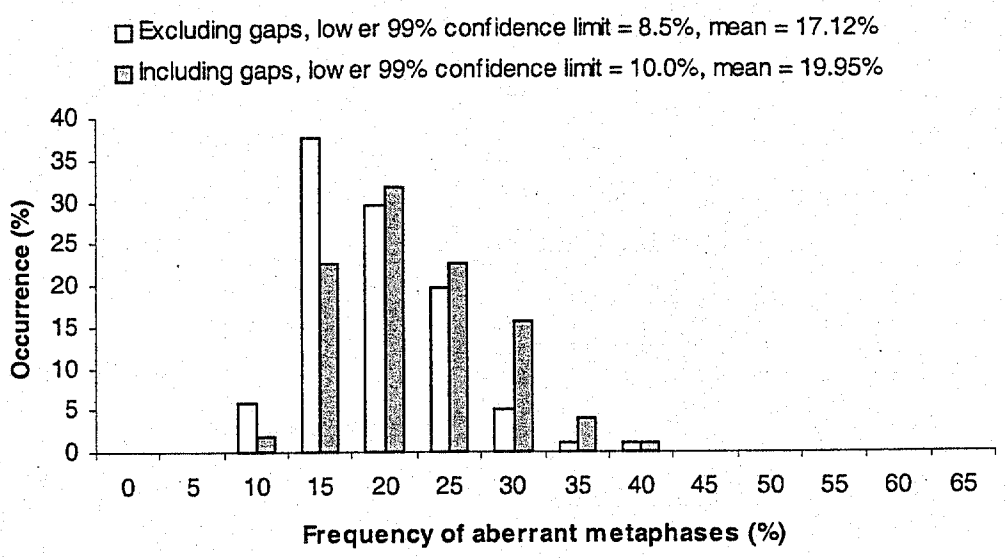


Historical negative control data (January 1999 - December 2001)
With S9 mix

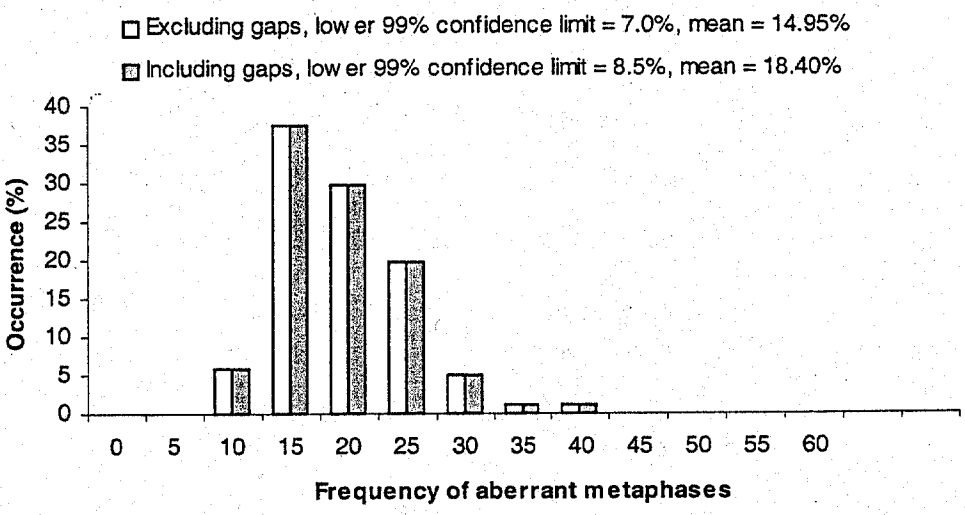


APPENDIX 2

Historical positive control data (January 1999 - December 2001)
Without S9 mix



Historical positive control data (January 1999 - December 2001)
With S9 mix



APPENDIX 3

APPARATUS FOR VAPOUR/GAS PHASE EXPOSURE OF CULTIVATED MAMMALIAN CELLS

FIGURE 1

Culture bottle (volume 160ml)

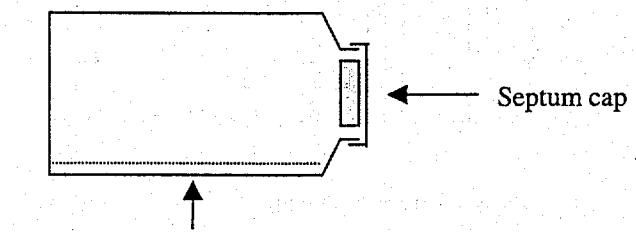
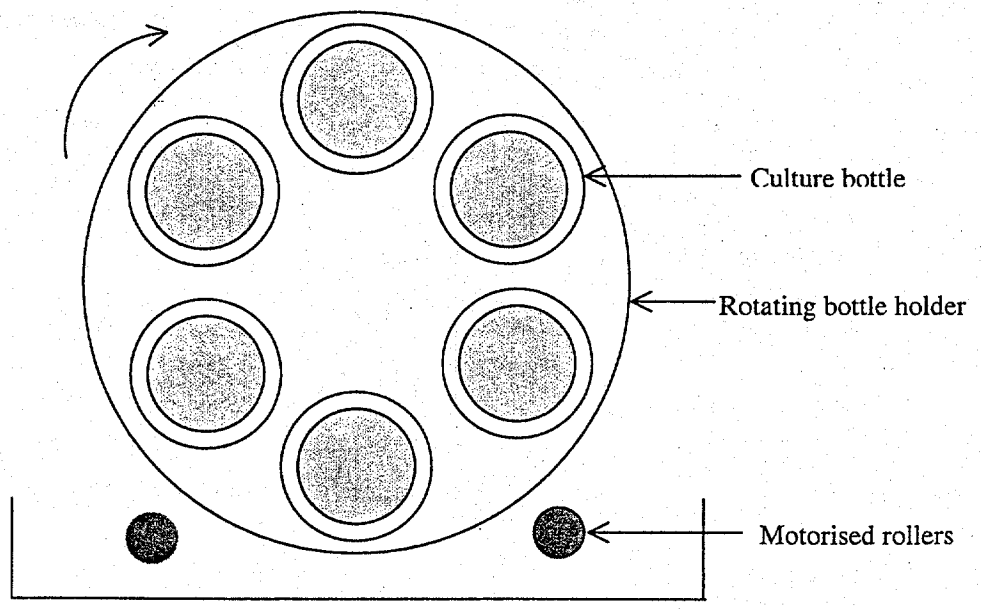


FIGURE 2

Roller apparatus



APPENDIX 4

Eye Research Centre GLP Compliance Statement, 2001

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY

Huntingdon Life Sciences
Eye Research Centre
Eye
Suffolk
IP23 7PX

TEST TYPE

Analytical Chemistry
Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys/Chem Testing
Toxicology

DATE OF INSPECTION

29th January 2001

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger J. Alexander
3/4/01

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

Enquiry number: 23923E

Huntingdon Life Sciences

T-7661.3

PROTOCOL

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST

IN HUMAN LYMPHOCYTES

Sponsor

3M Center
3M Corporate Toxicology
Building 220-2E-02
St Paul
MN 55133-3220
USA

Research Laboratory

Huntingdon Life Sciences Ltd
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

000075

Enquiry number: 23923E

Huntingdon Life Sciences

PROTOCOL APPROVAL

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST

IN HUMAN LYMPHOCYTES

C. Atterwill
Management
Huntingdon Life Sciences Ltd

Date 18/07/01

3M Center
Sponsor

Date 31/7/01

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Study Director approval of the protocol is given on the study details page of the protocol once such details have been established and agreed. The completed page will be issued prior to the start of the study.

000076

Enquiry number: 23923E

Huntingdon Life Sciences

STUDY DETAILS PAGE

Study number:	MIN/315
Study title:	*In vitro* mammalian chromosome aberration test in human lymphocytes
Test substance	
Identity:	Perfluorooctanesulfonyl Fluoride (POSF)
Lot number:	040227
Expiry:	Sponsor's responsibility; assumed stable for duration of study
Appearance:	Clear liquid
Storage conditions:	Room temperature
Purity/Assay:	>95.5%
Specific Gravity:	*ca* 1.8
Solvent:	To be determined
Stability of test substance formulation:	Not assessed in this study
Analysis of achieved concentration:	Not assessed in this study
Sponsor's Monitoring Scientist:	John Butenhoff
Head, Department of Genetic Toxicology:	Dr Gillian Clare
Study Director:	Ms Linda Allais
Person acting in the temporary absence of the Study Director:	Mr Lincoln Pritchard
Location of study:	Dept. Genetic Toxicology, Huntingdon Life Sciences Ltd., Eye, Suffolk, IP23 7PX, England
Proposed study dates:	
Start:	*ca* 14 January 2002
Completion:	*ca* 15 March 2002
Draft report:	Within four weeks of experimental completion

STUDY DIRECTOR APPROVAL OF PROTOCOL

Ms Linda Allais
Study Director
Huntingdon Life Sciences Ltd

10 January 2002
Date

000077

Enquiry number: 23923E

Huntingdon Life Sciences

CONTENTS

1. INTRODUCTION

The object of this study is to assess the mutagenic potential of the test substance in an *in vitro* cytogenetic test system using cultured human lymphocytes. This procedure complies with the following guidelines:

OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: *In Vitro* Mammalian Chromosome Aberration Test, Guideline 473.

US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5375 *In Vitro* Mammalian Chromosome Aberration Test. EPA 712-C-98-223.

2. BACKGROUND

Most chemical mutagens produce recognisable chromosomal damage expressed as structural aberrations. A well established test system using cultured human lymphocytes has frequently been used to observe these aberrations (Evans and O'Riordan 1975, Scott, Dean, Danford and Kirkland 1990). The aberrations are best observed at the metaphase stage of cell division, when the chromosomes are contracted. Division of the cells can be arrested at metaphase using the mitotic inhibitor, Colcemid®, which prevents formation of the mitotic spindle. The best estimate of the aberration frequency is at the first cell division after treatment since certain types of damage will be lost before subsequent cell divisions. The cultures will be harvested after a period of time which is approximately 1.5 times the cell cycle after initiation of treatment (usually 18 - 21 hours).

Many substances do not exert a mutagenic effect until they have been metabolised by enzyme systems that are not found in cultured cells. Therefore the cultures and test substance are incubated in both the absence and presence of a supplemented liver fraction (S9 mix) prepared from rats previously treated with a substance (Aroclor 1254) known to induce a high level of enzymic activity (Maron and Ames 1983, Natarajan *et al.* 1976).

3. EXPERIMENTAL PROCEDURE

Culture of lymphocytes

Blood will be taken from healthy male donors and diluted with tissue culture medium (RPMI 1640 containing 10% foetal calf serum, heparin and antibiotics). Lymphocytes, which do not normally undergo cell division, will be stimulated to do so by the addition of the naturally occurring mitogen, phytohaemagglutinin (Evans and O'Riordan 1975, Nowell 1960). The cultures will be prepared as 5 ml aliquots (0.4 ml blood: 4.5 ml medium: 0.1ml PHA solution) in sterile universal containers and incubated at 37°C. The cultures will be occasionally shaken to resuspend the cells.

Positive controls

In the absence of S9 mix

Identity:	Mitomycin C
Supplier:	BDH Biochemical or other suitable supplier
Appearance:	Blue powder
Solvent:	Water
Concentration:	0.1 - 1.6 µg/ml

In the presence of S9 mix

Identity:	Cyclophosphamide
Supplier:	Asta Medica Ltd. or other suitable supplier
Appearance:	White powder
Solvent:	Water
Concentration:	5 - 30 µg/ml

Preparation of S9 fraction

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Age:	7-8 weeks
Weight:	<300g

S9 fraction will be prepared from a group of usually *ca* 10 animals. Mixed function oxidase systems in the rat liver will be stimulated by Aroclor 1254. administered in an appropriate vehicle as a single intraperitoneal injection at a dosage of 500 mg/kg bodyweight. On the fifth day after injection, following an overnight starvation, the rats will be killed and their livers aseptically removed.

The following steps will be carried out at 0-4°C under aseptic conditions. The livers will be placed in 0.15 M KCl (3 ml KCl : 1 g liver) before being transferred to a homogeniser. Following preparation, the homogenate will be centrifuged at 9000 *g* for 10 minutes. The supernatant fraction (S9 fraction) will be dispensed into aliquots and stored at -80°C or below until required. All batches of S9 fraction will be tested for sterility and efficacy.

Preparation of S9 mix

S9 mix contains: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors will be filter-sterilised before use.

Culture treatment

Two sets of cultures will be established, one set to be treated in the absence of S9 mix, the other in its presence. All lymphocyte cultures will be incubated for approximately 48 hours, following stimulation with PHA, before exposure to the test substance. The cultures will be centrifuged and the cells will be resuspended in fresh culture medium (final volume 5 ml, containing S9 mix (1 ml) where appropriate). Atmospheres of the test material at concentrations of 1.25, 2.5, 5, 10, 20, 40 and 70% v/v (70% v/v is the maximum practicable concentration) will be established in sealed glass bottles (160 ml internal volume) with septum caps. Air will be withdrawn from each bottle and then an appropriate volume of test material liquid will be introduced using a syringe and needle, inserted through the septum cap, to produce atmospheres at the required concentrations. After evaporation of the volatile material and equilibration of the atmospheres at 37°C, the lymphocyte cultures (5 ml, containing S9 mix where appropriate) will be injected into the glass bottles. The glass bottles will then be incubated on their sides at 37°C in a roller apparatus which rotates the bottles once every eight minutes approximately. The lymphocytes coat the inside of the bottles and will be immersed in culture medium once every revolution and exposed directly to the treatment atmospheres for the rest of each revolution. This system allows maximum exposure to the treatment atmosphere, while maintaining good cell growth. Negative and positive control cultures will contain an atmosphere of air. Positive control cultures will be treated with Mitomycin C (in the absence of S9 mix) and cyclophosphamide (in the presence of S9 mix).

At the higher concentrations there is an increased risk of artefactual increases in aberrations due to osmotic effects (Galloway *et al.* 1987). Change of pH in the medium, indicated by a colour change, may also cause artefactual damage (Morita, Watanabe, Takeda and Okumura 1989, Kitching, Mason and Jones 1997). Accordingly, any such colour changes will be recorded. Treatment media may be retained for osmolality and pH measurement if deemed necessary. Duplicate cultures will be used for each treatment. S9 homogenate will be present in appropriate cultures at a final concentration of 5% v/v. All cultures will be identified using unique number/colour codes.

In the absence and presence of S9 mix, cultures will be incubated for three hours in the presence of the test substance. At the end of this three hour period, the cultures will be centrifuged and the cells will be cultured in fresh medium under an atmosphere of air in universal containers. The cultures will then be incubated until the scheduled harvest time (usually 18-21 hours).

Harvesting

Two hours before the cells are harvested, mitotic activity will be arrested by the addition of Colcemid® at a final concentration of 0.1 µg/ml. The cells will then be harvested by centrifugation and treated with a hypotonic solution (0.075M KCl) to cause swelling. The cells will be fixed in cold methanol:glacial acetic acid (3:1 v/v) and stored at 4 °C until the slides are prepared.

Slide preparation

A homogenous cell suspension will be prepared and aliquots of this cell suspension will be dropped onto pre-cleaned microscope slides and left to air-dry. The prepared slides will be stained in 10% Giemsa, left to air-dry and then mounted in DPX. All of the remaining cell cultures will be stored at approximately 4°C until the slide analysis has been completed.

Microscopic examination

The prepared slides will be examined by light microscopy. The incidence of mitotic cells per 1000 cells will be assessed (except for positive control treated cultures).

From these results, at least three dose levels for subsequent metaphase analysis will be selected. The highest dose level will usually be that causing a depression in mitotic index of at least 50% when compared with the solvent control level or, if the test substance lacks toxicity, the highest concentration used in the test. Intermediate and low concentrations will be selected at the discretion of the Study Director. The intermediate dose level will usually show some evidence of toxicity, whereas the lower dose level will be non-toxic. When this range of toxicity occurs at test concentrations which have precipitate visible in the culture medium at the end of the treatment period, one or more of the concentrations selected for metaphase analysis will be above the solubility limit. If a clear toxicity pattern is not apparent, the study may be repeated. The dose levels used in the repeat test may differ from those used in the initial assay. The concentration of positive control compounds selected for analysis will be the lowest concentration dosed unless a preliminary scan of metaphase figures indicates an insufficient level of aberrant cells.

Following mitotic index assessment but prior to metaphase analysis all slides will be coded. Metaphase figures will be examined by light microscopy and the incidence of chromosomal aberrations per 100 metaphase spreads per culture will be scored. Nomenclature of chromosomal damage will be based on the system proposed by the ISCN (ISCN 1985). Only cells with 44-48 chromosomes will be analysed. The vernier readings of aberrant metaphase figures will be recorded.

The incidence of polyploid metaphase cells will be determined for negative control cultures and cultures treated with the highest dose level of the test substance used in the analysis for chromosomal aberrations. Five hundred metaphase cells (where possible) will be analysed for polyploidy.

Despite evidence showing some gaps to be real discontinuities in DNA (Heddle and Bodycote 1970, Satya-Prakash, Hsu and Pathak 1981) the exact nature of the chromatid gap is uncertain and may not be a true indicator of damage. Because of this uncertainty results will be analysed both including and excluding gaps. A gap will be defined as a non-staining region, smaller than the width of a chromatid, where there is minimal misalignment of the chromatid.

Second test

Following the results of the first test, a second independent test will be conducted. The test treatment period for the set of cultures to be treated in the absence of S9 mix will be dependent on the results of the first test, i.e. 3 hour treatment in both the presence and absence of S9 mix. If a negative result is obtained a different treatment regime will be employed. Normally this will entail continuous treatment of cultures in the absence of S9 mix, pulse treatment will again be used for cultures in the presence of S9 mix. The need to vary treatment conditions will be evaluated on a case-by-case basis at the discretion of the Study Director. All cultures will be harvested at the same time as in the first test. The procedures used are as those described previously. The choice of test concentrations may vary from the first test e.g. a narrower range may be used on the basis of the toxicity observed in the first test.

4. ASSESSMENT OF RESULTS

An assay is considered to be acceptable if the negative and positive control values lie within the current historical control range.

The numbers of aberrant and polyploid metaphase figures in each treatment group will be compared with the solvent control value using a one-tailed Fisher's test (Fisher 1973). This is a useful test for analysing data when comparing two independent samples. It is used when the observed events fall into one or other of two mutually exclusive classes. The test determines whether the two groups differ in the proportions with which they fall into the two classifications.

The test substance will be considered to be positive if the following conditions are met:

Statistically significant increases in the frequency of metaphases with aberrant chromosomes (excluding gaps) are observed at one or more test concentration.

The increases exceed the negative control range of this laboratory, taken at the 99% confidence limit.

The increases are reproducible between replicate cultures.

The increases are not associated with large changes in osmolality of the treatment medium or extreme toxicity.

Evidence of a dose-relationship will be considered to support the conclusion.

A negative response will be claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies is observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

5. REPORTING

The report will contain details of the test substance, methodology, results and interpretation of the data. The tabulated results of mitotic index and metaphase analysis data for each culture will be shown. Good Laboratory Practice and Quality Assurance statements will be included.

6. MAINTENANCE OF RECORDS

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

7. GOOD LABORATORY PRACTICE

The study will be conducted in compliance with the principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

8. QUALITY ASSURANCE

The following will be inspected or audited in relation to this study.

Protocol Audit	:	Study specific protocol.
Process based inspections	:	Routine and repetitive procedures will be inspected on representative studies, not necessarily on this study.
Report Audit	:	The draft report and study data will be audited before issue of the draft report to the Sponsor.

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections which will be reported to appropriate Company Management only.

9. HEALTH AND SAFETY

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1994, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

10. REFERENCES

EVANS, H.J. and O'RIORDAN, M.L. (1975) Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. *Mutation Research*, **31**, 135.

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MARON, D.M. and AMES, B.N. (1983) Revised methods for the Salmonella mutagenicity test. *Mutation Research*, **113**, 173.

MORITA, T., WATANABE, Y., TAKEDA, K. and OKUMURA, K. (1989) Effects of pH on the *in vitro* chromosomal aberration test. *Mutation Research*, **225**, 55.

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NOWELL, P.C. (1960) Phytohaemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. *Cancer Research*, **20**, 462.

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Study Number: MIN/315

Protocol Amendment Number: 1

Huntingdon Life Sciences

T-7661.3

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES

Total number of pages: 4

Number of pages for internal distribution: 4

Study Director : Ms. Linda Allais, DEA Tox., DESS Pharm. Vet., France.

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 17.1.2002
(Study Director)

000087

For the Sponsor

Approved by: John R. Butterhoff Date: 21.1.2002

Study Number: MIN/315

Protocol Amendment Number: 1

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES

Reasons for amendments :

To rectify the following paragraphs, as detailed on the Protocol:

Original statement:

Study Details page (page iii)

Stability of test substance formulation: Not assessed in this study

5. Reporting

The report will contain details of the test substance, methodology, results and interpretation of the data. The tabulated results of mitotic index and metaphase analysis data for each culture will be shown. Good Laboratory Practice and Quality Assurance statements will be included.

8. Quality Assurance

The following will be inspected or audited in relation to this study.

Protocol Audit : Study specific protocol.

Study Number: MIN/315

Protocol Amendment Number: 1

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES

Revised statement:

Study Details page (page iii)

Stability and homogeneity of test substance formulation: Not assessed in this study

5. Reporting

The report will contain details of the test substance, methodology, results and interpretation of the data. The tabulated results of mitotic index and metaphase analysis data for each culture will be shown. Good Laboratory Practice and Quality Assurance statements will be included.

In the absence of ongoing communications, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional cost.

Upon study completion, two types of report are issued:

Draft report: Following QA audit, for review by the Sponsor

Final report: After approval by the Sponsor

Reports will be supplied on A4 paper and the following number of copies will be supplied:

Draft report: 1 unbound (double sided)

Final report: 1 bound (double sided with original signatures)

1 unbound (single sided)

Any additions or corrections to an authorised final report will be documented as a formal amendment to the report.

8. Quality Assurance

The following will be inspected or audited in relation to this study.

Protocol Audit : Study specific protocol and amendments.